

~~translational activities. The invention allows the monitoring of the location of such~~
intracellular specific binding partners over time and in response to stimuli, such as test chemicals. The spectroscopic probes can be used for screening a test chemical for activity. The present invention also includes cells and transgenic organisms comprising the intracellular specific binding partner, wherein the specific binding partner can bind with the spectroscopic probe/ligand conjugate.

Please amend the specification as follows:

At page 7, please replace the paragraph at lines 4-19 with the following paragraph:

B2
FIG. 3 represents site-specific labeling of CHO cells. **FIG. 3A** is a confocal image of CHO cells transfected with the plasma membrane-targeted sFv vector and shows plasma membrane staining by phOx-rhodamine. **FIG. 3B** is a confocal fluorescence image of the cells in **FIG. 3A** stained with a fluorescein-labeled anti-c-myc antibody. **FIG. 3C** shows the brightfield image of the cells in **FIG. 3A**. **FIG. 3D** shows a fluorescence image of CHO cells with Golgi-targeted sFv in the presence of 10 nM phOx-Bodipy[®]F1. **FIG. 3E** shows the same cells as in **FIG. 3D** in the presence of 500 nM phOx-ethanolamine and 10 nM phOx-Bodipy[®]FL. **FIG. 3F** is a fluorescence image of a cell with ER-targeted sFv in the presence of 10 nM phOx-Bodipy[®]. **FIG. 3G** shows immunostaining of Golgi-sFv transfected cells with fluorescein labeled, anti-c-myc antibody. **FIG. 3H** shows immunostaining of Golgi-sFv transfected cells with rhodamine-labeled anti-mouse antibody directed against a mouse 58k protein antibody. **FIG. 3I** shows immunostaining of ER-sFv transfected cells with rhodamine-labeled anti-mouse antibody directed against an anti-c-myc antibody. **FIG. 3J** shows immunostaining of ER-sFv transfected cells with fluorescein-labeled concavalin A. The scale bar represents 10 micrometers.

At pages 40-41, please replace the paragraph at line 14 of page 40 to line 3 of page 41 with the following paragraph:

B3 A flexible aliphatic linker was added to 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (phOx) (Sigma) by reaction of 50 mg phOx with 14.3 μ l 1,5-diaminopentane (Aldrich) in 2.5 ml acetone for 1 h. The di-substituted aminopentane was precipitated by addition of two volumes of 50 mM borate buffer (pH 9.2) leaving the product in solution. A more rigid trans-cyclohexane linker was added to phOx by reaction of trans-1,4-diaminocyclohexane (Aldrich) (5.1 mg in 0.5 ml of DMSO) and phOx (10 mg in 0.5 ml acetone) for 2 h. The di-substituted diaminocyclohexane was precipitated by addition of an equal volume of water, leaving the monosubstituted diaminocyclohexane in solution. PhOx-Bodipy[®] Fl was prepared by reaction of excess Bodipy[®] FL succinimidyl ester (Molecular Probes) with 1 mM of phOx-aminopentane in borate buffer for 2 h. The product was obtained as a precipitate. PhOx-tetramethylrhodamine was prepared by reaction of excess tetramethylrhodamine succinimidyl ester (Molecular Probes) with 5 mM phOx-aminocyclohexane in borate buffer for 6 h. The product was obtained as a precipitate. PhOx-fluorescein was prepared by reaction of equimolar amounts of phOx (0.5 mg in 25 μ l acetone) and fluorescein cadaverine (Molecular Probes) (1 mg in 50 μ l dimethylformamide) for 1 h. PBS was added, unreacted phOx was removed by hexane extraction, and the product was extracted with butanol. PhOx-ethanolamine was prepared by reaction of 1 mg phOx with 0.3 μ l ethanolamine in 10 ml ethanol for 1 h. All reactions were conducted at room temperature. Products were judged to be greater than 95% pure by TLC and compounds were confirmed by mass spectrometry using well-known methods.

At pages 41-42, please replace the paragraph at line 24 of page 41 to line 10 of page 42, with the following paragraph:

B4 A cell labeling method was developed that combines the site specificity conferred by genetically encoded protein targeting sequences with the spectral and indicator properties of fluorophores. The strategy is to express a high affinity specific binding partner at a specified

intracellular location to trap a conjugate of an indicator linked to a specific ligand (**FIG. 2A**).

B4 We chose a single-chain antibody (Chesnut, supra, 1996) (sFv) as the specific binding partner, and a high affinity hapten (phOx) as the ligand. Although many receptor-ligand pairs are possible, the antibody-hapten pair used here was chosen because of the simple ligand-probe chemistry and high affinity interaction without interference from cellular factors. For sFv targeting, cells are transfected with cDNAs encoding sFv in fusion with Golgi, ER or plasma membrane targeting sequences. Fluorophore-hapten conjugates were added to the extracellular solution at low concentrations, diffused to sites of sFv expression, and bound to the sFv. Conjugates of different indicator, spectral and linker properties were synthesized (**FIG. 2B**), including phOx-Bodipy[®] FL (green fluorescent, flexible linker), phOx-fluorescein (green fluorescent, pH-sensitive, flexible linker), and phOx-tetramethylrhodamine (red fluorescent, rigid linker). The flexible linkers were designed to permit stacking of the unbound hapten with its covalently attached fluorophore to form a dark (non-fluorescent) complex.

At page 43, please replace the paragraph at lines 4-19 with the following paragraph:

B5 The various sFv targeting constructs and phOx conjugates were studied in CHO cells. **FIG. 3A** shows a fluorescence image of living cells expressing the sFv at the plasma membrane and stained with phOx-rhodamine. A plasma membrane staining pattern was found. **FIG. 3B** shows staining of sFv in the same cells with a fluorescein-labeled anti-c-myc antibody. Comparison with **FIG. 3A** demonstrates that only sites of sFv expression were significantly labeled with phOx-rhodamine. There was no significant staining of adjacent cells that did not express sFv (**FIG. 3C**). **FIG. 3D** shows specific phOx-Bodipy[®] staining of Golgi. Staining was reversed by addition of 1 μ M phOx-ethanolamine (**FIG. 3E**). **FIG. 3F** shows phOx-Bodipy[®] staining of ER, seen as a characteristic reticular pattern. The high expression level of the sFv, the relatively high affinity of the hapten/sFv, and the low fluorescence of the unbound conjugate allowed images to be obtained in the presence of < 10 nM concentrations of unbound conjugate with little contribution from free conjugate. For quantitative measurement

BS of organelle pH, the free dye was washed out of the bathing solutions. Leakage out of the Golgi, which required dissociation from the sFv and diffusion through lipid membranes and unstirred layers, had a half time of tens of minutes.

On page 43, please replace the paragraph at lines 20-31 with the following paragraph:

B6 The subcellular location of expressed sFv was confirmed by immunofluorescence. Cells transfected with the Golgi-sFv construct showed perinuclear staining by a fluorescein-labeled, anti-c-myc antibody (**FIG. 3G**) which co-localized with staining by antibodies against the Golgi marker 58k protein (**FIG. 3H**). Cells transfected with the ER-sFv construct showed a reticular staining pattern with the c-myc antibody (**FIG. 3I**) which co-localized with staining by fluorescein labeled concavalin A, an ER marker (**FIG. 3J**). The membrane permeability of the conjugates was high enough to load cells by incubation at 37°C for 4 hours for phOx-fluorescein, 2 hours for phOx-rhodamine, or 10 minutes for the less polar phOx-Bodipy®. Cells could be loaded at 4°C, indicating that the conjugate entered the cells primarily by transmembrane diffusion and not by endocytosis. These results demonstrate the selective targeting of fluorescent probes to expressed sFv in living cells.

In the Claims

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Please cancel claim 7.

Please amend the following claims to appear as follows:

1. (Amended) A method for localizing a probe, comprising:

a) contacting a sample comprising a cell expressing a single chain antibody with a membrane permeant probe/ligand conjugate, said probe/ligand conjugate comprising:

- i) a probe moiety,
ii) a ligand that can bind with said single chain antibody, and